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Award Number: DAMD17-00-1-0116

TITLE: Endothelial Progenitors as Vectors for Systemic Gene Therapy of Breast Cancer

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REPORT DATE: August 2003

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	August 2003	Annual (10 Jul 02-9 Jul 03)

4. TITLE AND SUBTITLE
Endothelial Progenitors as Vectors for Systemic Gene Therapy of Breast Cancer

5. FUNDING NUMBERS
DAMD17-00-1-0116

6. AUTHOR(S)
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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES
Original contains color plates. All DTIC reproductions will be in black and white.

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT (Maximum 200 Words)

One promising new treatment modality for breast cancer is the application of vector-mediated gene therapy. A noted problem with many vector systems however, including both viral and non-viral vectors, used for gene therapy is the lack of efficient and targeted delivery to the primary tumor and disseminated metastases. To address this issue, we propose the use of $CD34^+$ and/or $Flk\text{-}1^+$ endothelial progenitor cells (EPCs), which have the propensity of homing to sites of neovascularization. Key to the success of this approach is efficient genetic modification of the EPCs. In this regard, we have shown previously that $CD34^+$ EPCs are efficiently transduced using live-viral vectors with relatively low doses and associated toxicity. We hypothesize that the modified EPCs can, after intravascular injection, localize into sites of tumor neovascularization and deliver therapeutic payloads. Further, the natural targeting capacity of EPCs will allow their use vectors for gene therapy of both local and disseminated breast cancer.

14. SUBJECT TERMS
Endothelial Progenitor Cells, Live-Viral Vectors, Adenovirus

15. NUMBER OF PAGES
16

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

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Abstracts and Personnel Associated With This Project

Abstracts

Li H, Kawakami Y, Curiel DT, and **Blackwell JL.** 2002. $CD34^+$ Endothelial Progenitors As Cellular Vehicles For Gene Therapy of Breast Cancer. Department of Defense Breast Cancer Era of Hope Meeting. Orlando, FL. (Poster)

Blackwell JL. 2002. $CD34^+$ Endothelial Progenitors As Cellular Vehicles For Gene Therapy of Breast Cancer. 2002 Department of Defense Breast Cancer Era of Hope Meeting. Orlando, FL. (Oral presentation)

Blackwell JL, Kawakami Y, Li H and Curiel DT. 2002. $CD34^+$ Endothelial Progenitors As Cellular Vehicles For Oncolytic Adenovirus Anti-Tumor Therapy. 5^{th} Annual Meeting of the American Society of Gene Therapy, Boston MA. (Oral presentation).

Personnel

Dr. Hui Li (Research Associate)
Jill Nagle (Research technician)

Introduction

The development of resistance to radiation and chemotherapeutic agents that cause DNA damage is a major problem for the treatment of breast cancer [1], which argues for the development of new therapeutic agents that can either augment the effects of radiation and chemotherapy or that can be applied as an adjunct or alternative treatments. One promising new treatment modality is the application of vector-mediated gene therapy. A noted problem with many vectors, including both viral and non-viral vectors, used for gene therapy is the lack of efficient and targeted delivery to the primary tumor and disseminated metastases. To address this issue, we propose the use of $CD34^+$ and/or $Flk\text{-}1^+$ endothelial progenitor cells (EPCs), which have the propensity of homing to sites of neoangiogenesis. Key to the success of this approach is a vector system for the efficient genetic modification of the EPCs. In this regard, we have shown previously that $CD34^+$ EPCs are efficiently transduced using live-viral vector with relatively low doses and associated toxicity. One promising new treatment modality for breast cancer is the application of vector-

mediated gene therapy. A noted problem with many vector systems however, including both viral and non-viral vectors, used for gene therapy is the lack of efficient and targeted delivery to the primary tumor and disseminated metastases. To address this issue, we propose the use of $CD34^+$ and/or $Flk\text{-}1^+$ endothelial progenitor cells (EPCs), which have the propensity of homing to sites of neovascularization. Key to the success of this approach is the efficient genetic modification of the EPCs. In this regard, we have shown previously that $CD34^+$ EPCs are efficiently transduced using live-viral vectors with relatively low doses and associated toxicity. We hypothesize that the modified EPCs can, after intravascular injection, localize into sites of tumor neovascularization and deliver therapeutic payloads. To test this hypothesis, we proposed to demonstrate that the EPCs (1) localize to sites of neovascularization in a murine model, (2) express virally-encoded transgenes and (3) effect therapeutic activity at tumor engraftment sites. Importantly, the natural targeting capacity of EPCs will allow their use vectors for gene therapy of both local and disseminated disease and establish a new paradigm for the treatment of breast cancer.

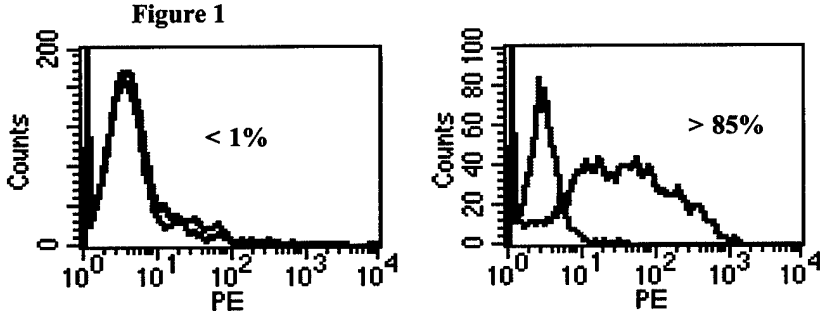
Body

On 9/14/00 the Principle Investigator responsibilities for this project were reassigned to Dr. Jerry L. Blackwell. I maintained an interest during the early developmental phases of the project [3, 4] and enthusiastically endorsed taking over the project when Dr. Jesus Gomez-Navarro left the University of Alabama and could no longer service the grant. Since taking over the Principle Investigator responsibilities I have assigned a highly experienced research assistant, Dr. Hui LI, to work on this project. Dr. Hui had ~50% effort dedicated to this project. In addition, Dr. Yosuke Kawakami, a talented post-doctoral fellow working in the Principle Investgator's lab, has also joined the project. Dr. Kawakami's primary role is to evaluate the *in vivo* translational

components of the project and had 25% effort dedicated to the project.

As discussed in the 2001 Annual Report, one of our first objectives was to develop a protocol for isolation of blood-derived CD34+ EPCs. We noted at that time that we would need populations of the CD34+ EPCs on a more frequent basis than what was originally anticipated. For this reason we investigated the use of fresh leukoconcentrates, or "buffy coats", that were readily obtainable from a local community blood bank at a nominal fee (i.e., $25/unit). We determined that the buffy coats, which are the equivalent to ~200 ml of whole blood, contain 10-100 x 10^6 total peripheral blood mononuclear cells (PBMC). Using an immunomagnetic positive selection procedure, we were able to isolate CD34+ EPCs from these PBMCs. Figure 1 shows the purity of CD34+ EPCs before (left) and after (right) isolation from PBMCs. Before purification < 1% of the cells are CD34+, yet after purification > 85% of the cells are CD34+. Between 2-5 x 10^6 CD34+ cells can be isolated from 1 buffy coat, which was plenty for the planned series of *in vitro* and *in vivo* studies. **The ability to obtain a highly enriched CD34+ EPC population from a readily available blood source has been a significant accomplishment in the progression of this project.**

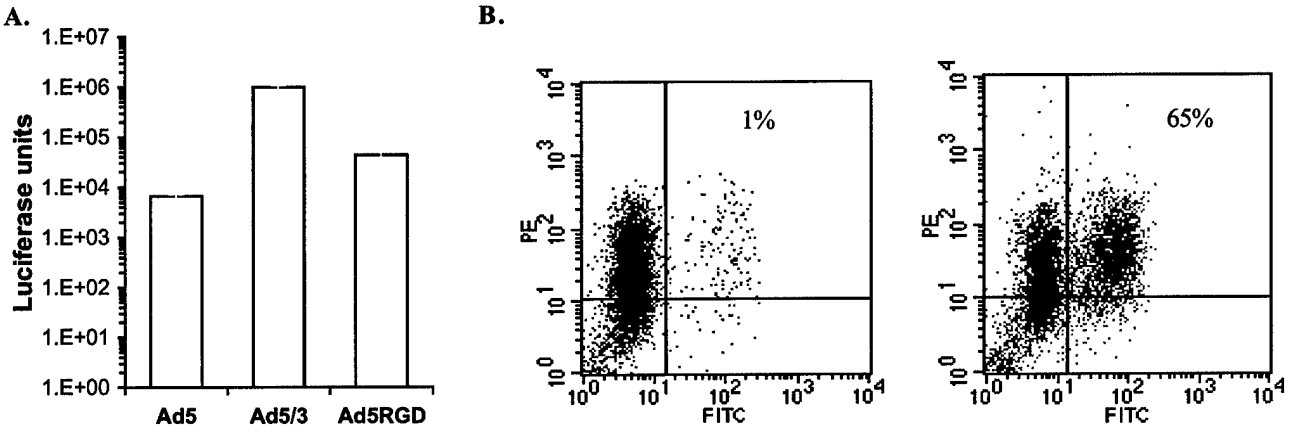
Figure 1



Also as noted in previous Annual reports, the ability to upscale herpesvirus production was a significant challenge. Sufficient amounts of herpesvirus were achievable for *in vitro* experiments, but the amounts needed for *in vivo* studies were very difficult to produce. Because of our experience using the adenovirus (Ad) as a gene transfer vector, we began a parallel series of pilot experiments to investigate the use of the Ad vector for gene transfer into CD34+ EPCs. We knew from previous

experience that upscaling would not be a problem if the Ad vector could be used for genetic modification of the CD34+ EPCs. In the first pilot experiment we observed that the Ad serotype 5 (Ad5) vector very inefficiently infected CD34+ EPCs, suggesting that CD34+ EPCs express no or low levels of the Ad5 receptor. Next we evaluated gene transfer using two novel Ad5 vectors, Ad5/3 and Ad5RGD, whose native tropism had been modified. The Ad5/3 vector infects cells through the Ad serotype 3 receptor and the Ad5RGD vector infects cells through RGD-binding integrins, such as the $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins. Figure 2A compares the efficiency of luciferase reporter gene transfer using the Ad5, Ad5/3 and Ad5RGD vectors at equal multiplicities of infection (MOI; MOI = 100). The Ad5/3 vector transduced the CD34+ EPCs >2 log factors better than Ad5. The Ad5RGD vector transduced the CD34+ EPCS ~1 log factor better than Ad5. We next evaluated the frequency of infection using flow cytometric analyses (Figure 2B). As shown in the upper-right quadrants in Figure 2B, approximately 1% of the CD34+ EPCs are infected by Ad5 (left) compared to 65% by Ad5/3

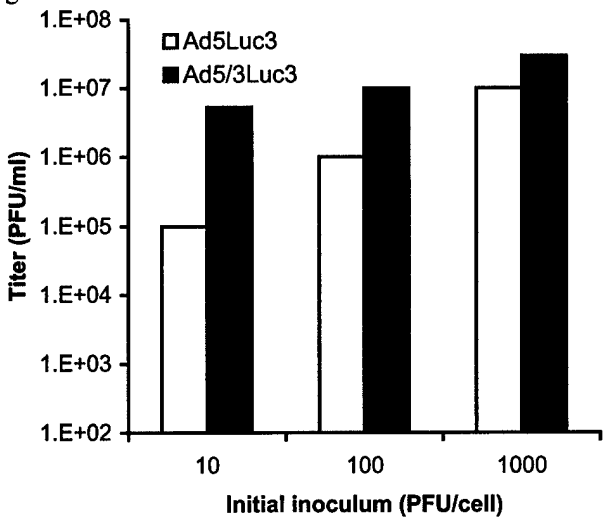
Figure 2



(right). This particular experiment was done using an MOI of 10. Using slightly higher MOIs (i.e., 25-50), we were able to quantitatively infect the entire CD34+ cell population (data not shown). **This series of experiments strongly suggests that the Ad vector, most notably the Ad5/3 vector, is a viable alternative as a gene transfer vector for genetic modification of CD34+ EPCs.**

Ad vectors have been extensively studied for gene therapy applications. Some of the noted advantages over other live-viral vectors include (i) the ability to easily grow and purify high titered Ad vector preparations, (ii) the Ad vector transduces both dividing and nondividing cells, and (iii) that compared to many other viral and most nonviral vectors, Ad vectors transduce a wide range of cell types. Considering these advantages and the results shown in Figure 2A and 2B, we decided to further investigate the use of tropism-modified Ad vectors for gene transfer to CD34+ EPCs. In this regard, one extremely promising area in gene therapy research is the use of oncolytic Ad vectors that selectively replicate in tumor tissues. These conditionally-replicative Ad (CRAd) vectors induce oncolysis in tumor tissue, but spare normal tissues [2]. We therefore sought to "load" CD34+ EPCs with tropism-modified CRAd vectors. The hypothesis is that the CD34+ EPCs will deliver their oncolytic payload to the tumor tissue vasculature where the CRAd would replicate. For this approach to succeed, it is necessary that the CD34+ EPCs support Ad replication, which has not been reported to date. To investigate this question, CD34+ EPCs were infected with replication-competent Ad5 or Ad5/3 vectors. Forty-eight hours later, the cells were harvested and amounts of *de novo* Ad vector production was measured by plaque assays on HEK293 cells (Figure 3). Infection by both Ad5 and Ad5/3 vectors resulted in *de novo* virus production. As predicted, the Ad5/3 infection resulted in much higher amounts of virus production. **This is the first report of the ability of CD34+ EPCs to support a productive Ad infection. Importantly, this experiment establishes the concept**

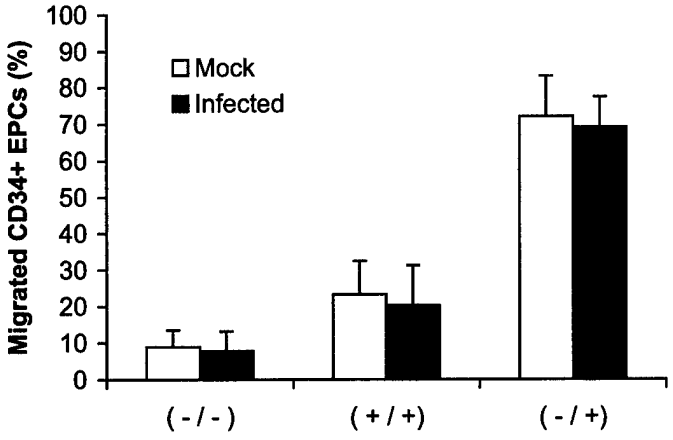
Figure 3



that CD34+ EPCs may be useful vehicles in the application of oncolytic virotherapy.

As just described above, one of our downstream goals is to use CD34+ EPCs for delivery of oncolytic Ad vectors to sites of tumor angiogenesis where the viral vector would subsequently replicate and destroy the tumor blood supply. For such a system to work, the infection of the CD34+ EPCs should not perturb the intrinsic ability to the CD34+ EPCs to home to the tumor in response to angiogenic signals, such as vascular endothelial growth factor (VEGF). VEGF is a known chemoattractant for CD34+ EPCs [5, 6]. We therefore developed an *in vitro* migration model to test whether infection by replication-competent Ad vectors affected the ability of the CD34+ EPCs to migrate to VEGF. Briefly, 24 after infection mock- or Ad5/3-infected CD34+ EPCs were placed in the top well of a cell migration chamber. As shown in Figure 4, VEGF was added neither the top nor bottom well (-/-), both the top and bottom wells (+/+), or only to the bottom well (-/+). Six hours later, the numbers of CD34+ EPCs that had migrated to the lower well were determined by a trypan blue exclusion assay. As shown in Figure 4, in the absence of VEGF in both the top and bottom well (-/-), only a small fraction of the CD34+ cell population migrated to the low well. When VEGF was present in both the top and bottom wells, a slightly higher number of cells migrated to the lower chamber. When VEGF was present only in the lower well nearly 75% of the CD34+ cells migrated to the lower well. Importantly, pre-infection with the replication-competent Ad vector did not significantly alter the migration activity of the CD34+ cells. **This experiment demonstrates that Ad infection of CD34+ cells does not perturb**

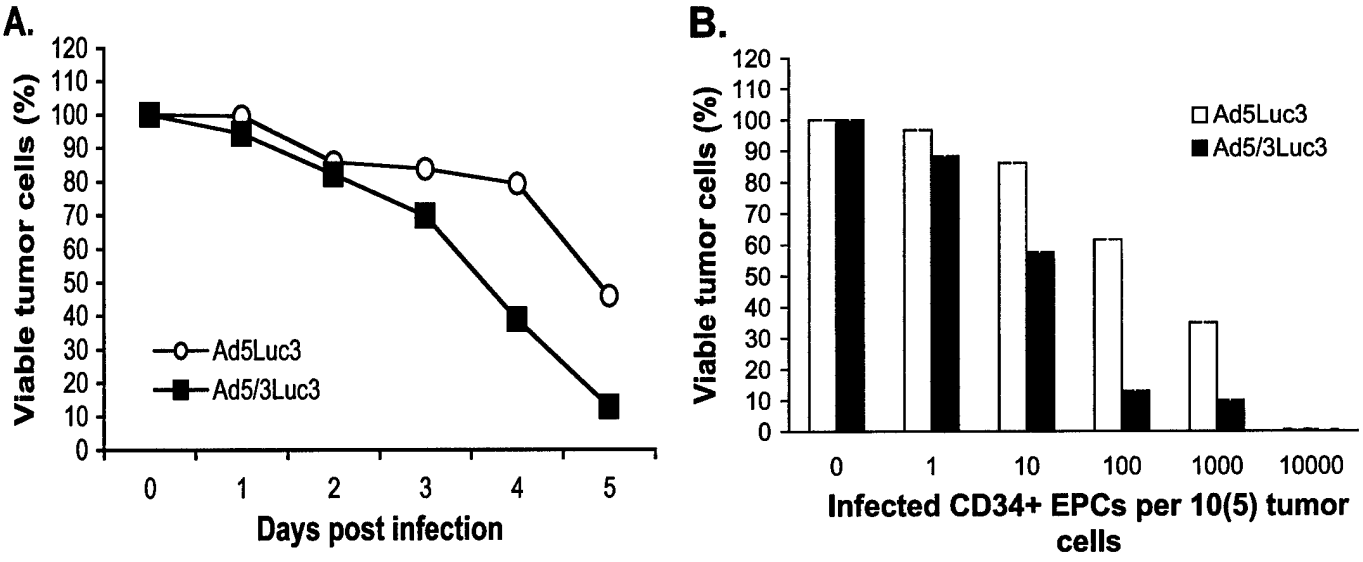
Figure 4



their migratory response to tumor-associated angiogenic signals. In addition, these results suggest that the CD34+ cells can be loaded with the oncolytic Ad vectors and deliver the viruses to tumor site *in vivo*.

Based on the previous results, we next investigated whether CD34+ EPCs loaded with oncolytic Ad vectors could be used therapeutically to kill tumor cells in an *in vitro* cell migration-cytolysis model. CD34+ EPCs were mock-infected or infected with either replication-competent Ad5 or Ad5/3 vectors. Uninfected or infected CD34+ EPCs were then placed in the top well of a chemotaxis chamber. Breast cancer cells were placed in the bottom well of the chemotaxis chamber. In addition, vascular endothelial growth factor (VEGF) was supplemented to the media in the bottom well of the chemotaxis chamber. The CD34+ EPCs were allowed to migrate to the bottom chamber for 6 h, after which the top well of the chemotaxis chamber was removed. The number of tumor cells killed by the release of oncolytic Ad vectors relative to the uninfected control was determined for the next 5 days (Figure 5A). The breast cancer tumor cells were efficiently killed in both groups, with the Ad5/3-infected CD34+ EPCs being more effective. This experiment was repeated with a total of 4 different breast cancer cell lines, all of which gave similar results. We also determined the approximate number of infected CD34+ EPCs needed to

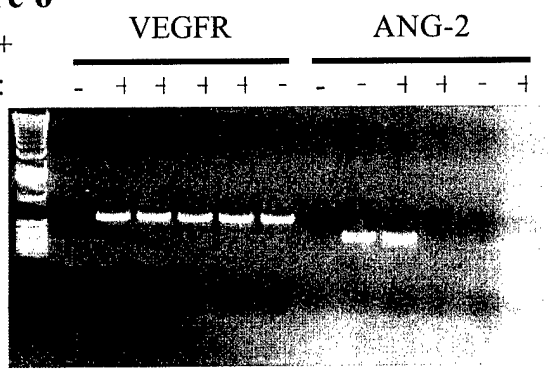
Figure 5



kill a fixed number of breast cancer cells (Figure 5B). In this experiment, different numbers of Ad5- or Ad5/3-infected CD34+ EPCs (i.e., 0-10000) were mixed with 100,000 breast cancer cells. Five days later the number of remaining breast cancer cells was determined. As shown in Figure 5B, between 1,000 and 10,000 Ad5-infected CD34+ EPCs and between 100 and 1,000 Ad5/3-infected CD34+ EPCs were required to kill approximately 90% of the breast cancer cells. **Collectively, these two experiments demonstrated that Ad-infected CD34+ EPCs can efficiently migrate to angiogenic signals and subsequently deliver the oncolytic virus resulting an in effective anti-tumor activity.**

The previous experiments establish the key proof-of-principle concepts that formed the impetus for moving to an *in vivo* murine model. The first objective was to determine if human CD34+ EPCs homed to tumor sites after being injected intravenously in mice. Briefly, human MCF-7 breast cancer cells were implanted subcutaneously on the flanks of athymic nude miceand then 12 days later CD34+ EPCs were injected intravenously. Fourteen days later, the tumors were harvested, total RNA was extracted and RT-PCR was performed using VEGFR and ANG-2 primers (Figure 6). These two genes have been used previously to detect the presence of engrafted human CD34+ EPCs [5]. In this experiment, all the tumors (5 of 5) in mice that received CD34+ EPCs expressed VEGFR and 3 of 5 tumors had detectable ANG-2 expression. None of the tumors that did not receive CD34+ EPCs had detectable VEGFR or ANG-2 expression, suggesting that the CD34+ EPCs were responsible for expression of these two markers. **Importantly, this experiments demonstrated that human CD34+ EPCs can home to human breast cancer xenografts in an *in vivo* murine model.**

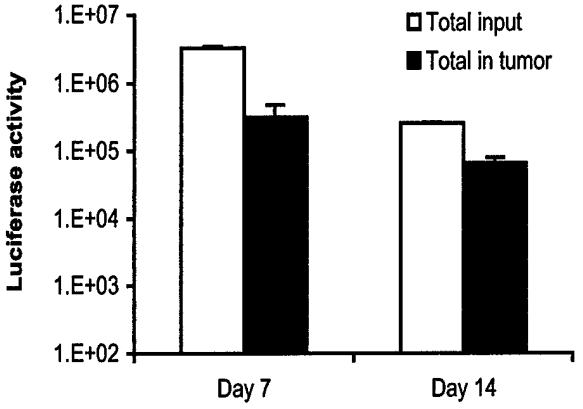
Figure 6



We next determined whether genetically modified human CD34+ EPCs could home to human breast cancer xenografts *in vivo*. An

experiment was performed exactly as described in the previous paragraph except that the CD34+ EPCs were infected with a luciferase-expressing Ad5/3 vector prior to intravenous infusion of 10^6 CD34+ EPCs. Seven and 14 days later the tumors were harvested and luciferase activity was measured (Figure 7). For comparison, luciferase activity was measured from Ad5/3-infected CD34+ EPCs maintained for the same periods of time. As evidenced by the luciferase activity in the tumors, approximately 10% of the total input CD34+ EPCs had reached the tumor site on Day 7 and Day 14. This suggests that approximately 100,000 infected CD34+ EPCs had reached the tumor site following infusion. There was a noticeable depreciation of luciferase activity on Day 14 compared to Day 7, which is consistent with what is understood about Ad gene expression. **This is the first ever demonstration of CD34+ EPC-mediated delivery of Ad to tumor sites *in vivo*.**

Figure 7



We next evaluated whether CD34+ EPCs loaded with oncolytic vectors could be used therapeutically to treated breast cancer xenografts in the murine model. This experiment was performed exactly as described above except that the CD34+ EPCs were infected with the replication-competent Ad5/3 vector prior to infusion. In this series of experiments we were unable to demonstrate a therapeutic effect. We believe the reason for this is due to untoward killing of the CD34+ EPCs by the oncolytic Ad vector prior to getting to the tumor site. We knew from the *in vitro* experiments that Ad replication killed the CD34+ EPCs in approximately 5 days. To address this issue, we are currently evaluating conditionally-replicative Ad (CRAd) vectors whose oncolytic activity can be regulated. One such CRAd vector that we are currently testing is regulated by the hypoxia response element. Our preliminary data suggests that the CRAd does not

replicate and kill the CD34+ EPCs under normoxia condition, whereas it does under hypoxia conditions. This would be an elegant solution to the problem currently faced with delivery of oncolytic Ad vectors to the tumor site. While the infected CD34+ EPCs is circulating in the blood (normoxia), the virus would be quiescent. However, when the CD34+ EPCs reach the tumor bed and engraft (hypoxia), the virus will be activated. Similarly, a tetracycline-inducible oncolytic Ad system is also under evaluation. Lastly, we are also attempting to load the CD34+ EPCs with an Ad vector that expresses imaging gene (i.e. somatostatin), which will provide a unique non-invasive method for detecting both local and disseminated breast tumors.

Key Research Accomplishments

- Validated protocol development for obtaining a highly enriched $CD34^+$ EPCs preparation from human peripheral blood mononuclear cells
- Established that the Ad vector system for genetic-modification of CD34+ EPCs may have important translational implications
- Demonstrated that tropism-modified Ad vectors can efficiently infect and replicate in CD34+ EPCs with low toxicity
- Demonstrated that CD34+ EPCs loaded with therapeutic agents can efficiently kill cancer tumor cells
- Shown that the genetic loading process does not interfere with CD34+ EPC migration functions in an *in vitro* chemotaxis model
- Generated data suggesting that CD34+ EPCs are capable of migrating and engrafting into human breast cancer xenografts in an *in vivo* murine model
- Demonstrated that Ad-infected CD34+ EPCs can engraft into human breast cancer xenografts in an *in vivo* murine model

Reportable Outcomes

- Invited to present the findings of the current study as an oral presentation at the 2002 American Society of Gene Therapy meeting in Boston MA. The title of the presentation was "CD34+ Endothelial Progenitors as Cellular Vehicles for Oncolytic Adenovirus Anti-Tumor Therapy".

- Invited to present the findings of the current study as an oral presentation at the 2002 Era of Hope DOD Breast Cancer Research Program's Meeting in Orlando FL. The title of the presentation is "CD34+ Endothelial Progenitors as Cellular Vehicles for Gene Therapy of Breast Cancer".

- Two post presentations at national science conferences.

- Manuscript in preparation entitled "Endothelial Progenitors as Cellular Vehicles for Oncolytic Adenovirus Anti-tumor Therapy" for submission to Cancer Research.

- Pilot project funding has been awarded through the UAB Breast Cancer SPORE mechanism based on work supported by this award.

- The "Cell Vehicle Working Group" has been created at the UAB Division of Human Gene Therapy to advance discoveries using this type of vector delivery/targeting system. The Cell Vehicle Working Group meets monthly to discuss project development and currently includes 6 members, which are Jerry Blackwell (P.I.), David Curiel (Division Director), Yosuke Kawakami (Post doctoral fellow), Laurisa Pereboeva (Group leader), Hui Li (Researh Assistant), and Jill Nagle (Graduate Research Assistant).

Conclusions

Important advancements have been made in the development of a readily available source of human CD34+ EPCs, which was a significant problem in the early phases of the project. We can now produce CD34+ EPCs on an "as-needed" basis. In addition to the herpesvirus vector system, we have also established a second live-viral vector system using Ad to genetically modify CD34+ EPCs. A strong argument can be made for using the Ad vector rather than the herpesvirus vector system, which includes both considerations of production and downstream translational issues. Most importantly, the project is now entering into the final *in vivo* testing phase where the real utility of the CD34+ EPCs delivery system will be rigorously evaluated. Whereas this is the most challenging element of the project, it is also the most exciting. Our preliminary data strongly suggests that this will be an achievable goal using conditionally-replicative Ad vectors. Continuing work will be pursued in that direction.

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